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TITLE: Crosstalk Between Cancer Cells and Bones Via the Hedgehog Pathway
Determines Bone Metastasis of Breast Cancer

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The Hh pathway has been extensively studied in development and recently has also been shown to be activated in a variety of cancer types, thus making it a putative therapeutic target. Our preliminary data indicated that OPN is a transcriptional target of Gli1. Gli1 is a transcription factor of the Hedgehog (Hh) pathway. Research in our laboratory indicates that OPN is under direct control of the Hh pathway as seen by the effect of agonistic Hh pathway ligands and the Hh pathway inhibitor, cyclopamine.
Moreover, we find that the Hh ligands, Shh and Ihh can stimulate differentiation of osteoblasts. The OPN-containing conditioned medium from the MDA-MB-435 cells is also able to potentiate differentiation of osteoblasts.

15. SUBJECT TERMS
hedgehog, osteopontin, breast cancer, osteoblasts

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INTRODUCTION
Subject of research
The levels of OPN are significantly elevated in the tumors and plasma of patients with metastatic breast cancer and are notably associated with decreased patient survival. Tumor cells upregulate OPN synthesis and secretion by osteoblasts leading to pathologic activation of osteoclasts, resulting in a net loss of bone. Thus, OPN enhances metastasis of breast cancer to bone.

Our preliminary data indicated that OPN is a transcriptional target of Gli1. Gli1 is a transcription factor of the Hedgehog (Hh) pathway. The Hh pathway has been extensively studied in development and recently has also been shown to be activated in a variety of cancer types, thus making it a putative therapeutic target.

Purpose of research
Through studies proposed in the grant, we will determine the role of the Hh pathway in regulating metastasis to bone.

*As stated in the grant, we **hypothesize** a novel crosstalk between breast cancer cells, osteoblasts and osteoclasts via the Hh pathway and OPN in the bone milieu that results in bone resorption in breast cancer.*

Scope of research
<u>Specific Aim 1:</u> Evaluate the involvement of the Hh pathway in bone metastasis of breast cancer.
<u>Specific Aim 2:</u> Perform a molecular analysis of the crosstalk between breast cancer cells, osteoblasts and osteoclasts involving the Hh pathway and OPN.
<u>Specific Aim 3:</u> Functionally analyze the hedgehog pathway in determining osteolytic lesions in metastatic breast cancer using mouse models of metastasis.

We anticipate that our work will bring forth markers viz. Gli1, Shh and OPN, which can predict the outcome of breast cancer, specifically with regard to its ability to metastasize to bone. In addition, the these molecules will also serve as new pharmacological targets for therapeutic intervention to prevent breast cancer from metastasizing to the bone.

BODY

Per the Statement of Work, we proposed that we would initiate the work with addressing the experiments proposed in Specific Aim 2.

Specific Aim 2: **Perform a molecular analysis of the crosstalk between breast cancer cells, osteoblasts and osteoclasts involving the Hh pathway and OPN**

Proposed cell lines to be used:

Breast cancer (BC) cells: Two metastatic breast cancer cell lines that make Shh, the transcription factor Gli1 and also produce OPN (i) MDA-MB-435 and (ii) MCF 10CA cl. A.

These cells are being studied for their effects on osteoblasts. In addition to these cells, we have acquired three more cell lines from Asterand Inc. These are breast cancer cell lines with a documented capability to metastasize viz. SUM44, SUM159 and SUM1315.

Osteoblasts (OB): Two osteoblast cell lines. (i) hFOB and, (ii) MC3T3-E1 pre-osteoblast cells. Both lines produce OPN.

We have acquired the MC3T3-E1 cells clones 14 and 24. Whereas, clone 14 has the capability to differentiate into osteoblasts in medium containing β-glycerophosphate and ascorbic acid, clone 24 is unable to differentiate. Both these cell lines were acquired from ATCC. We have used the MC3T3-E1 clone 14 cells in experiments (presented below).

Osteoclasts (OC): Osteoclasts (i) We will induce differentiation of the RAW264.7 cells of the macrophage/monocyte lineage to osteoclasts with M-CSF and RANKL and (ii) we will isolate stromal cells from the bone marrow of BALB/c mice and induce them to differentiate into mature osteoclasts in presence of M-CSF and RANKL over 6 days.

We have acquired the RAW cells and have assessed them for their expression of molecules of the Hh pathway and OPN (data presented below).

Task 1: will address the effect of breast cancer cells on osteoblasts.

Outcomes:
1. *We initiated this Task by first examining the expression of various molecules of the Hh pathway in breast cancer cell lines, osteoblasts and osteoclasts (Figures 1, 2 and 3). Specifically, we examined the expression of transcription factors Gli1 and Gli2 and the two ligands of the Hh pathway, Shh and Ihh.*
2. *We also assessed the expression of OPN by these cells. (Figure 3).*
3. *We assessed the effects of the conditioned medium of breast cancer cells on osteoblasts. Specifically, we evaluated the effects of the conditioned serum-free medium of the MDA-MB-435 cells on MC3T3-E1 clone 14 (Figure 14).*
4. *We anticipate that we will soon initiate studies to examine the interaction between these cells and osteoblasts.*

Task 2: will address the effect of breast cancer cells on osteoclasts.

Outcomes:
We have acquired the RAW264.7 cells and have assessed their expression of OPN and other molecules of the Hh pathway (Figures 1-3).
We anticipate that we will soon initiate studies to examine the interaction between these cells and osteoblasts.

Task 3: will address the effect of osteoblasts on osteoclasts.

Outcome:
We have yet to begin this aspect of Specific Aim 2.

Specific Aim 1: Evaluate the involvement of the Hh pathway in bone metastasis of breast cancer.

Task 1: Optimize conditions for staining the tumor tissue, minimizing the noise from the "surrounding normal" tissue. This will be done using archived tumor material from the Department of Pathology, University of South Alabama.

Outcomes:
We have standardized the immunohistochemistry conditions for staining OPN and Gli1 (Figure 13).

Tasks 2, 3 and 4: *Presently, we have initiated the procedure to obtain tissues from Pathology as well as from the NCI Tissue Microarray Facility. Our proposal to obtain tissues has been reviewed by the Co-operative Human Tissue Network and is pending some statistical clarifications.*
Once we obtain these slides, we will evaluate the expression of Gli1, Shh, Ihh and OPN in these tissues.

Specific Aim 3: Functionally analyze the hedgehog pathway in determining osteolytic lesions in metastatic breast cancer using mouse models of metastasis.

Task 1: We will use fluorescent GFP-tagged MCF10CA cl. A and MDA-MB-435 cells. Both these cells produce abundant OPN. We will clone 5 shRNAs targeting various regions of Gli1 mRNA into pSUPERIOR (Oligoengine). These will be evaluated by transient transfections for their ability to 'silence' Gli1 expression.

Outcomes:

1. We bought the plasmid pSUPERIOR from Oligoengine. We also ordered 3 duplex oligos that were predicted to target Gli1 for degradation.
2. These 3 duplexes were cloned into pSUPERIOR. We then tested the effects of the three shRNA constructs for their ability to silence the expression of endogenous Gli1. We have found that construct #1 is effectively able to silence Gli1 (Figure 12).
3. We have stably transfected this construct into 2 cell lines: MDA-MB-435 and SIM 1315. These cell lines have been chosed because oftheir ability to metastasize to the bone upon orthotopic injections into the mammary fat pads of athymic nude mice.
4. Presently, these transfected cell lines are in "selection" medium that contains puromycin so as to select for stable clones that have been silenced for Gli1.
5. We have confirmed that the transfection of construct #1 causes suppression of OPN expression. This is in complete agreement with our hypothesis and supports further studies.

Tasks 2, 3, 4 and 5: *These will be initiated and completed in due course after we have obtained and ascertained the cell lines that have been stably silenced for Gli1.*

In addition to the proposed studies, we have done some experiments in order to conclusively establish the relationship between the Hh pathway and OPN. These are presented below.

Specifically, we have used the following approaches:
1. *Treating the cells with the Hh pathway inhibitor, cyclopamine: As seen in Figures 4 through 9, treatment of cells with cyclopamine caused a decrease in the expression of OPN (WB: anti-OPN Ab).*
2. *Treatment of cells with cyclopamine causes a decrease in the activity of the OPN promoter (Figure 7).*
3. *The Hh ligands, Shh and Ihh are able to upregulate the activity of the OPN promoter (Figure 10).*
4. *Gli1 upregulates the activity of the OPN promoter (Figure 11).*

KEY ACCOMPLISHMENTS

✓ The Hh pathway regulates OPN
   o Treatment of cells with cyclopamine inhibits nuclear translocation of Gli1 concomitant with inhibition of OPN expression
   o Treatment of cells with Hh ligands, Shh and Ihh, activates OPN expression.
   o The transcription factor, Gli1, is able to upregulate expression of OPN.

✓ Treatment with conditioned medium from breast cancer cells potentiates the differentiation of osteoblasts.
   o Differentiation of osteoblasts is stimulated by the Hh ligands, Shh and Ihh.
   o The OPN from the secreted medium of breast cancer cells stimulates differentiation of osteoblasts.

✓ We have identified the shRNA that is effectively able to silence Gli1 expression. Development of stably-silenced cells is underway.

REPORTABLE OUTCOMES

August 2007   "Role of osteopontin in breast cancer tumorigenicity and metastasis" Presented at Gordon Research Conference on SIBLING proteins, New England, August 5-10, 2007.

February 2008   "Role of osteopontin in breast cancer tumorigenicity and metastasis" Presented at the Cell Biology and Neurosciences Journal Club series, University of South Alabama, Feb 6th, 2008.


CONCLUSION

We have conclusively shown that the Hh pathway stimulates the expression of OPN.

FIGURE 1: Expression of ligand, Indian hedgehog (Ihh) in various breast cancer cell lines, the osteoblast cell lines, hFOB and MC3T3-E1 and the osteoclast cell line, RAW264.7.

MCF10CA clone d
MDA-MB-231
MDA-MB-435
SUM159
MC3T3-E1
hFOB
RAW264.7

Ihh (45kDa)

Ihh (25kDa)

β-tubulin

Secreted Ihh (25kDa)
(concentrated 30X through a 10K cut-off column)
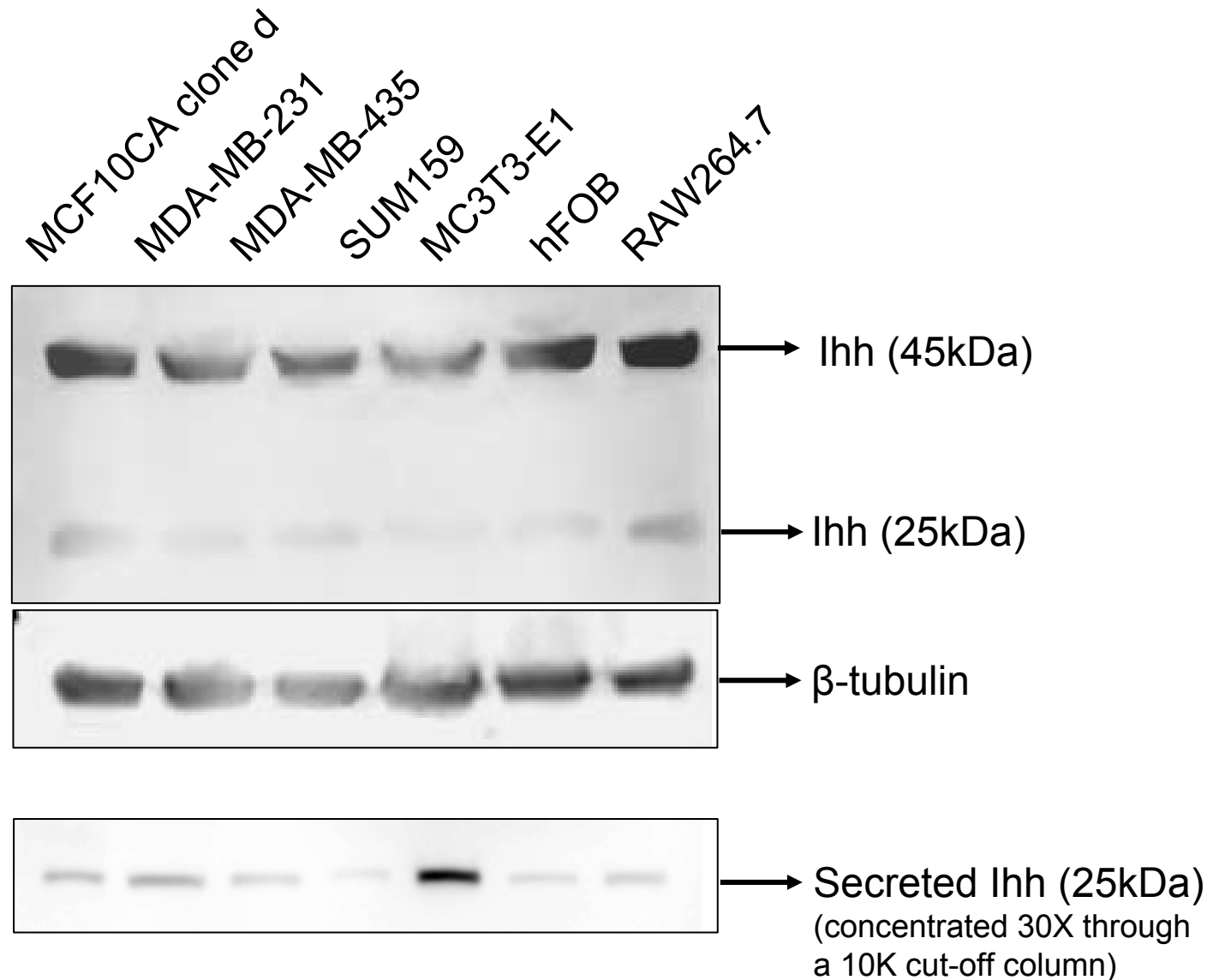
FIGURE 2: Expression of ligand, Sonic hedgehog (Shh) in various breast cancer cell lines, the osteoblast cell lines, hFOB and MC3T3-E1 and the osteoclast cell line, RAW264.7.
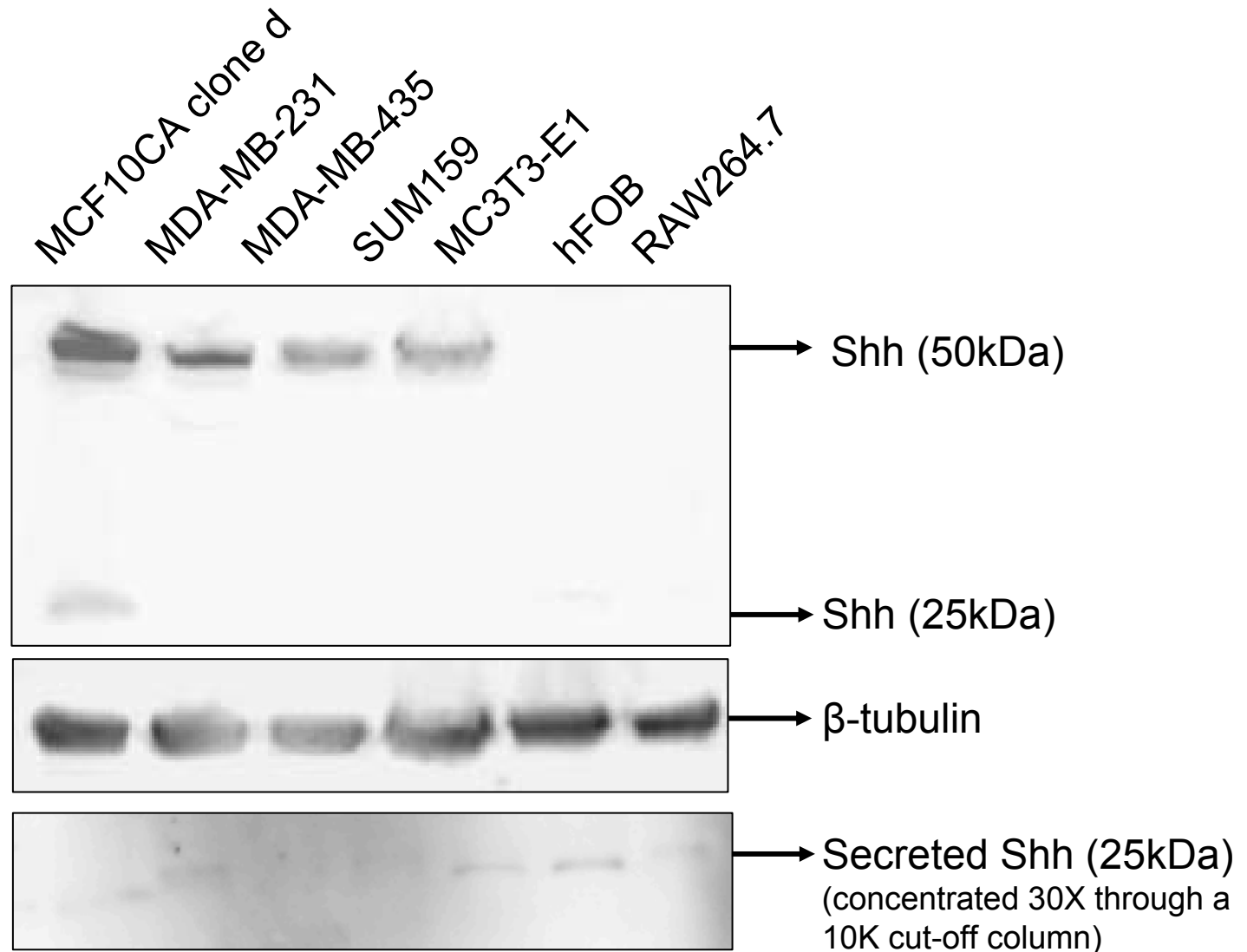
FIGURE 3: Expression of Gli1, Gli2 and OPN in various breast cancer cell lines and in the osteoblast cell line, hFOB.
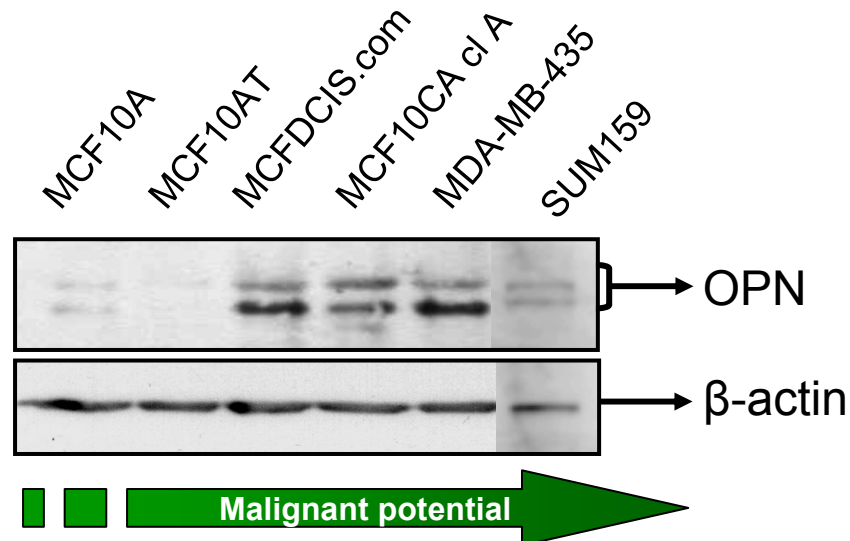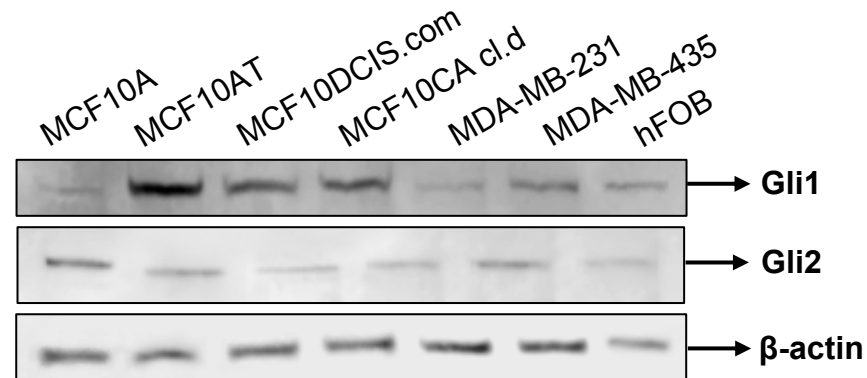
FIGURE 4: Cyclopamine treatment inhibits nuclear transport of Gli1 in MDA-MB-435 cells
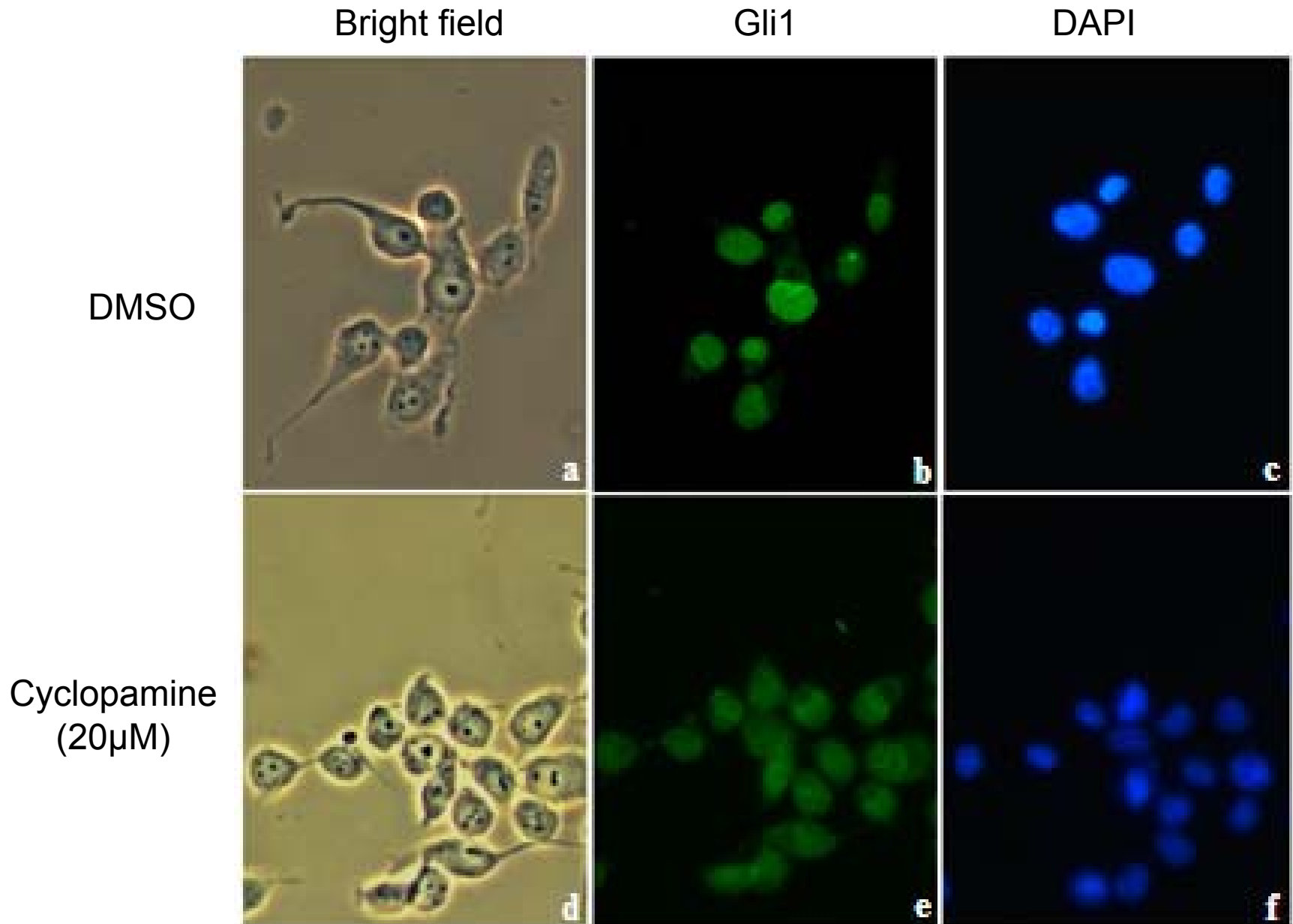
# FIGURE 5: Cyclopamine treatment inhibits nuclear transport of Gli1 in hFOB cells

	Bright field	Gli1	DAPI
DMSO			
Cyclopamine (20μM)			
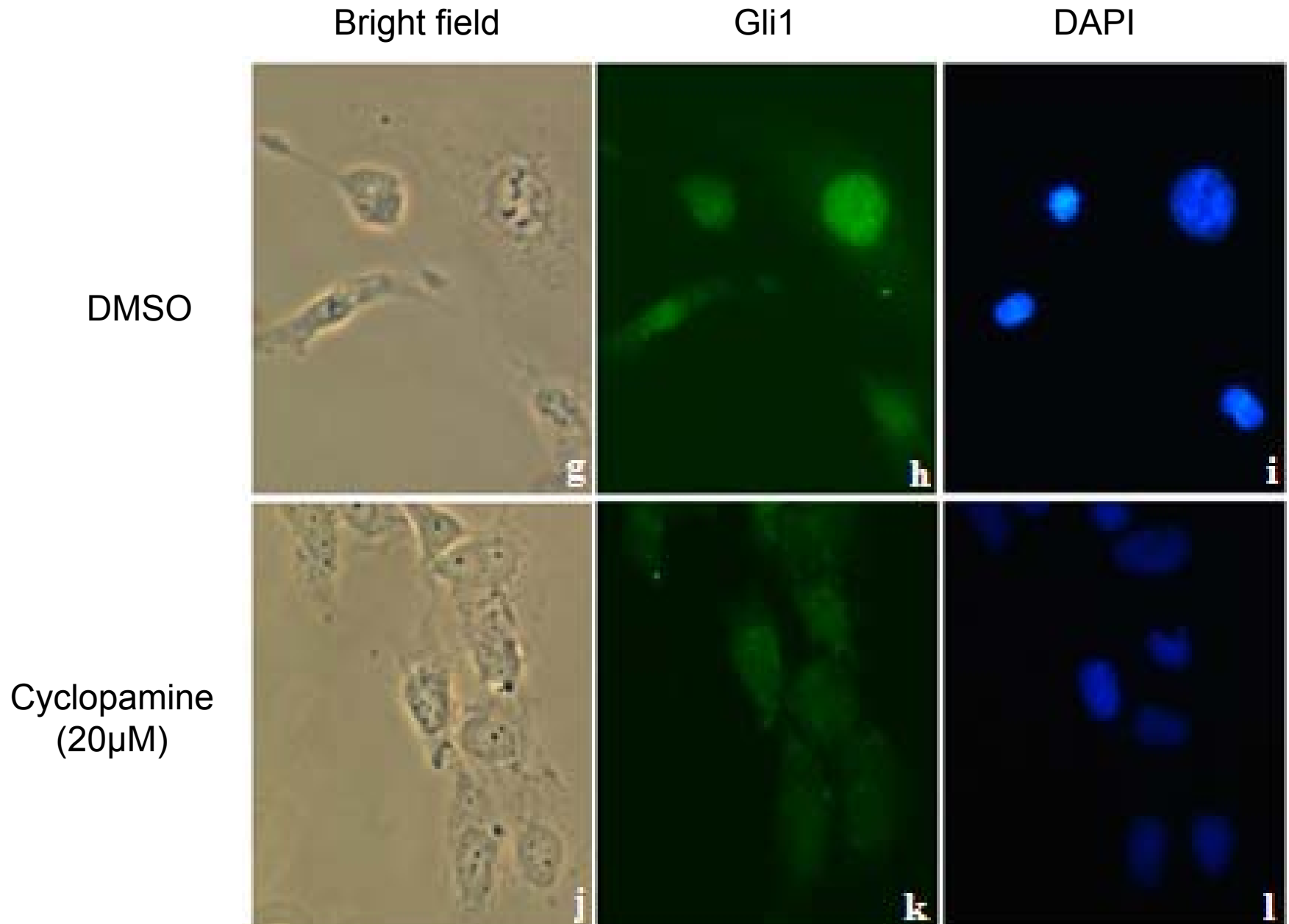
FIGURE 6: Cyclopamine treatment decreases levels of nuclear Gli1 concomitant with a decrease in nuclear Gli1
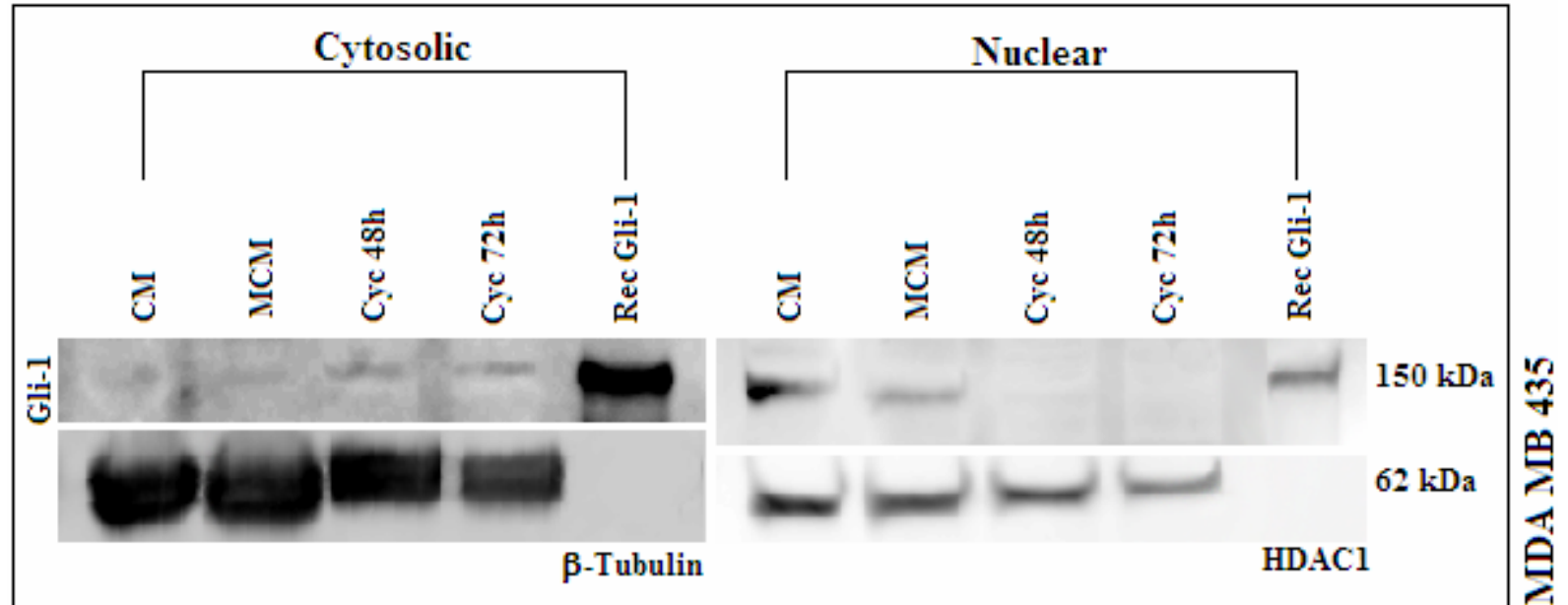
FIGURE 7: Cyclopamine treatment decreases expression of OPN
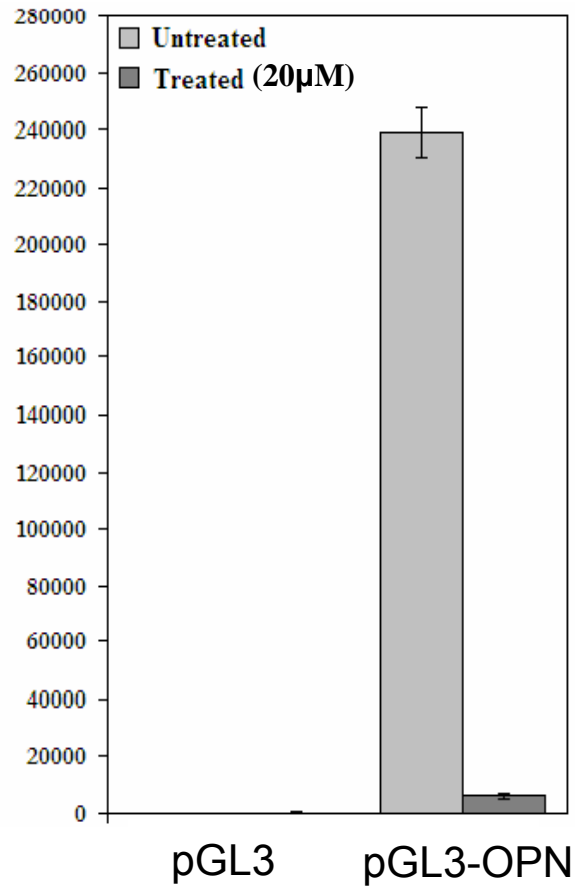
Luciferase reporter activity of OPN promoter; in presence or absence of cyclopamine
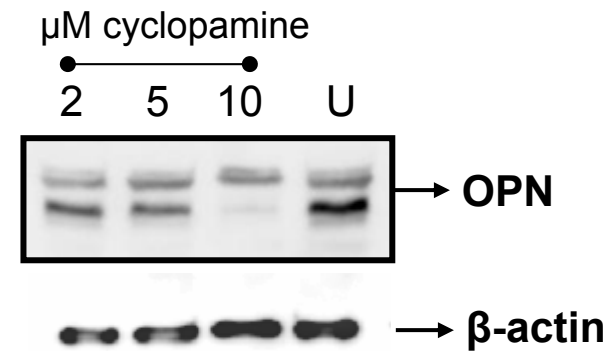
Western blot showing decreased OPN upon treatment with cyclopamine

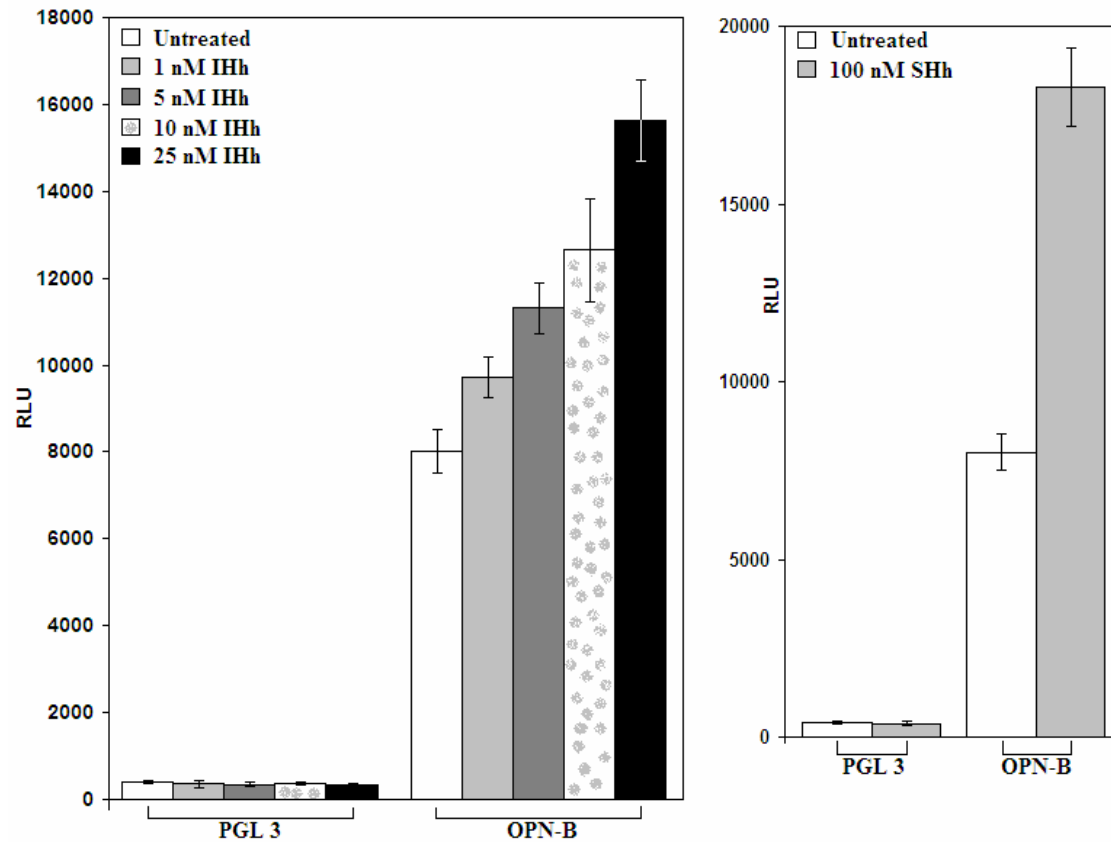**FIGURE 8: Cyclopamine treatment decreases secreted OPN**

12 hours   24 hours   36 hours   48 hours
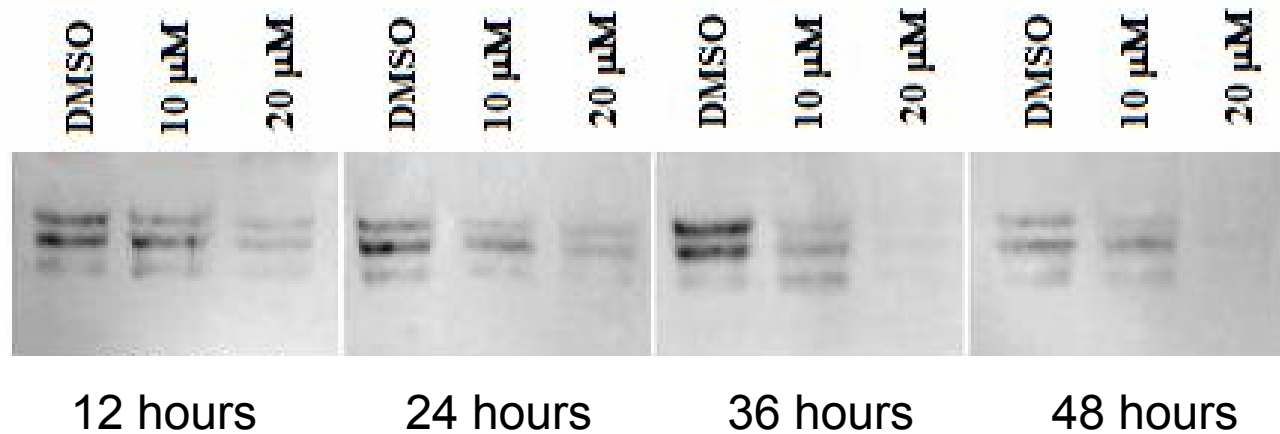
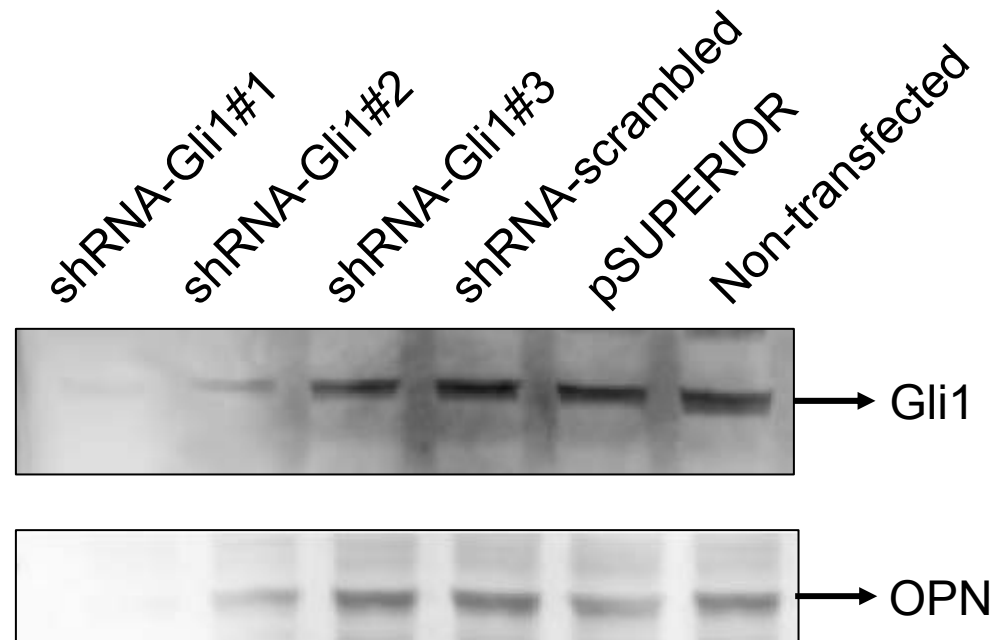FIGURE 9: Cyclopamine treatment decreases the pool of OPN mRNA
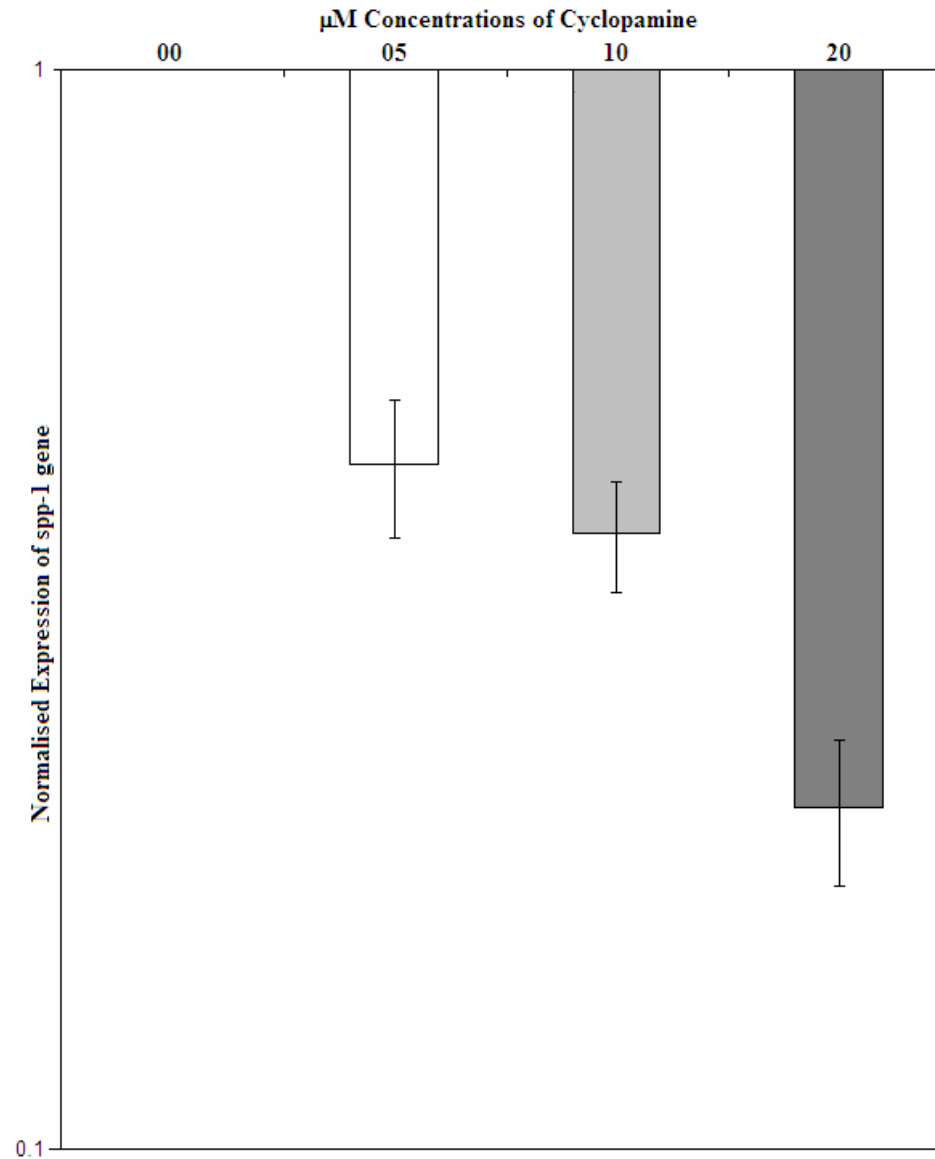
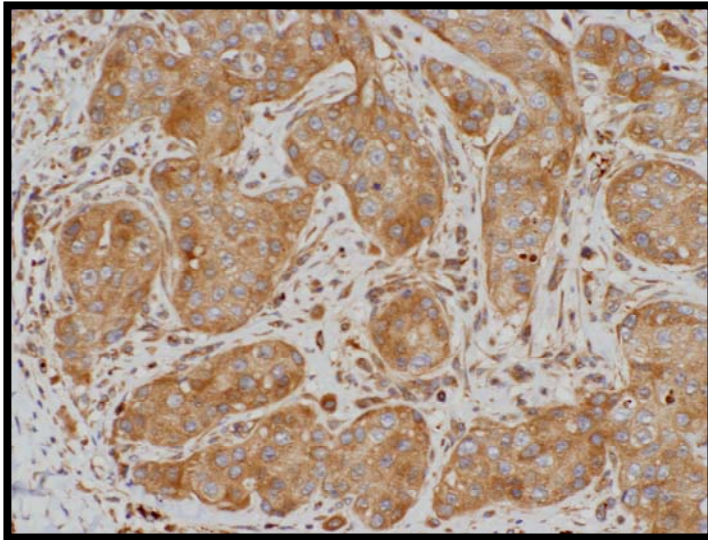**FIGURE 10: The Hh ligands, Ihh and Shh stimulate activity of OPN promoter**
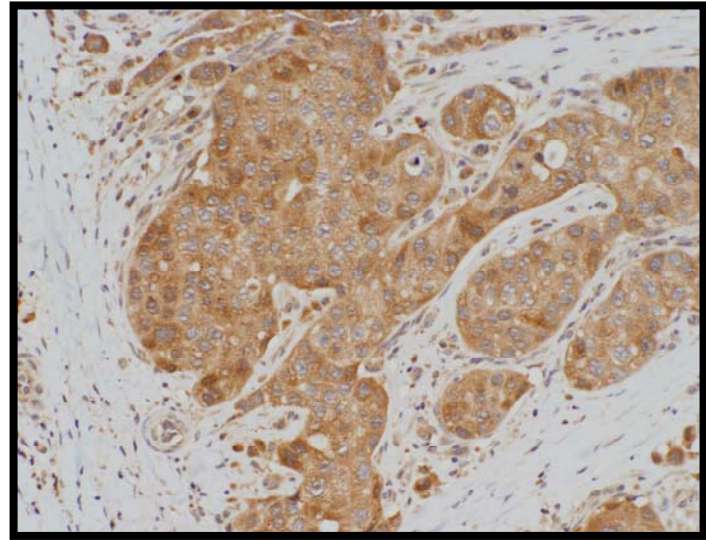
FIGURE 11: Gli1 activates OPN promoter activity
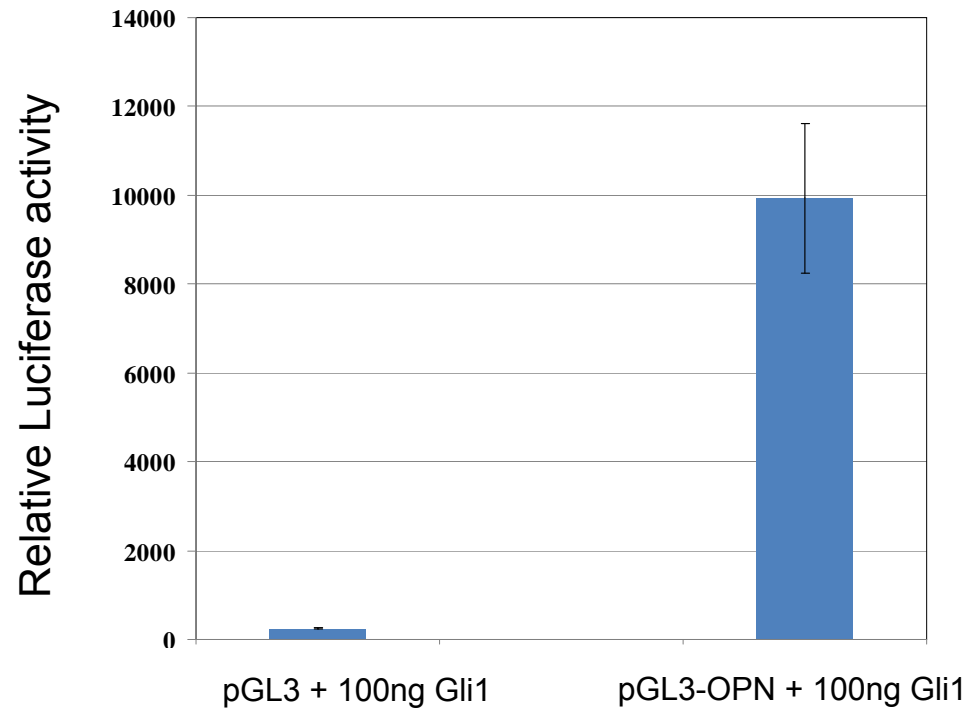
FIGURE 12: shRNA to Gli1 abrogates expression of OPN

FIGURE 13: Aggressive breast cancers show increased expresssion of Osteopontin and Gli1

OPN

Gli1

FIGURE 14: Addition of Ihh and Shh to differentiation medium (DM) potentiate differentiation of MC3T3-E1 cells. A 1:1 blend of DM and conditioned medium of MDA-MB-435 cells (435-SFCM) also stimulates differentiation of osteoblasts.